EFFECT OF JUVENILE HORMONE ON THE SYNTHESIS AND ACCUMULATION OF A SEX-LIMITED BLOOD PROTEIN IN THE POLYPHEMUS SILKMOTH

MARTIN BLUMENFELD[1] AND HOWARD A. SCHNEIDERMAN

Developmental Biology Center, Case Western Reserve University, Cleveland, Ohio 44106

Juvenile hormone (JH) plays a key role in insect development. As long as immature insects produce and release this agent, they do not mature. When they no longer release JH, or release it in small amount, they metamorphose. The effect of JH on the maturation of immature insects is seen when a JH extract (Williams, 1956), or pure JH (Röller *et al.*, 1967; Meyer *et. al.*, 1968) is injected into a pupa of the Polyphemus silkmoth, *Antheraea polyphemus*. When a normal pupa is allowed to develop in the absence of juvenile hormone, it molts into an adult moth about 18 days after the first signs of apolysis. However in a JH-injected pupa, adult development is blocked, and the pupa precociously molts into a so-called second pupa 10–12 days after the injection (Gilbert and Schneiderman, 1960). During the pupal-pupal molt, the insect's epidermal cells, instead of secreting adult cuticle, secrete a cuticle which closely resembles the original pupal cuticle. Also the morphogenetic death of many of the epidermal cells of the wings and antennae, which accompanies normal adult development, does not occur.

Because of its effects on metamorphosis, JH might be expected to influence the synthesis, appearance, or disappearance of specific proteins. The present report analyzes the effects of JH on the synthesis, release and accumulation of a specific protein found in the blood of female Polyphemus pupae.

Materials and Methods

Female pupae of *Antheraea polyphemus* that had been chilled at 2° C. for six months were removed from the cold and anesthetized with carbon dioxide. Each animal was bled through a cut in the facial region between the right wing and right antenna. About 20 μl. of blood was squeezed through this cut into a 0.5-ml. polyethylene microcentrifuge tube containing a few crystals of phenylthiourea (PTU) (Schmidt and Williams, 1953). Immediately after the blood was collected, the wound was sealed with melted paraffin. The blood samples were centrifuged for two minutes in a Beckman microfuge at 12,500 g and stored at $-10°$ C.

The animals were injected with a JH extract prepared according to the method of Gilbert and Schneiderman (1960). Some of the operated animals were injected with 75 JH units/g. pupal weight (*i. e.*, 150 mg./g. pupal weight of a 1:1

[1] Present address: Department of Zoology, The University of Michigan, Ann Arbor, Michigan 48104.

mixture of JH:peanut oil (PO)). Control animals were injected with 150 mg./g. pupal weight of PO, or were uninjected. Blood samples were collected from the same animals on consecutive days during the pupal-pupal molt and during the pupal-adult transformation. On each succeeding day, 5–10 μl. of blood was collected through the original wound. After each such operation, the wound was immediately resealed. JH-injected animals were bled every day until they molted into second pupae 8–10 days after the injection. Control animals were bled daily for 17 to 21 days. The fat body and ovaries were removed from all animals at the end of the experiment and were stored at −10° C.

The proteins in these blood samples were separated electrophoretically, and their relative concentrations were estimated by densitometry.

All samples were examined using high pH discontinuous electrophoresis as described by Davis (1964) and Ornstein (1964). Preliminary experiments, in which the gel concentrations and the amount of protein applied to each gel were varied, indicated that the desired proteins were best resolved by electrophoretically separating 0.5 μl. of blood in 4.5% gel. This quantity of blood was applied to the gel column as 10 μl. of a 1/20 solution of blood in insect Ringer solution (Ephrussi and Beadle, 1936) and separated electrophoretically.

Upper and lower gel solutions were purchased from Canal Industries, Silver Spring, Maryland. Gels were prepared according to the manufacturer's instructions. The upper and lower buffers were prepared by diluting a stock solution containing 6.0 g. Tris and 28.8 g. glycine per liter of distilled water 1:9 with distilled water.

Electrophoresis was carried out at 22–24° C. at 3 mA/gel until the bromphenol blue tracking dye reached the bottom of the lower gel, usually 50–60 minutes. Following electrophoresis, the gels were removed from their holders and stained for 30 minutes with a solution of 0.5% Buffalo Black in 7.5% acetic acid, and destained electrophoretically in 7.5% acetic acid.

The destained gels were scanned in a Joyce-Loebl densitometer at a 1:9 ratio (1 mm. of gel length = 9 mm. of record). The machine measured and recorded the density of the stained protein bands, and automatically integrated the area under the curve for each band. The densitometric data for Polyphemus blood proteins were compared with standard densitometric curves obtained with specific amounts of beef serum albumin, and the amount of protein in a given band was expressed as μg. of beef serum albumin equivalents (N. G. Patel, unpublished observations). These measurements were accurate to ±5%. The blood collected from three experimental animals and three control animals was analyzed in this way.

The fat body and ovaries of experimental and control animals were thawed, suspended in cold Ringer solution in microcentrifuge tubes containing PTU, and sonicated for five minutes in a Bronwill cup sonicator at maximum probe intensity. Microscopic examination revealed that this treatment ruptured all of the cells. The samples were then centrifuged at 12,500 *g* for five minutes. The low density fatty layer was skimmed from the top of each sample, and the supernatants were then separated electrophoretically.

In some experiments, fat body from female Polyphemus pupae was implanted into the abdomens of male pupae, which were allowed to develop into adults. Blood was collected from these animals, and analyzed electrophoretically.

Results

1. *Effects of various treatments on development*

The blood sampling procedure had no effect on development. Both experimental and control animals showed the first visible signs of development of apolysis two days after removal from the cold. Control animals completed their metamorphosis 20–22 days after removal from the cold, whereas experimental animals completed their pupal-pupal development 8–10 days after removal from the cold.

The second pupae were covered with a pupal cuticle. Their ovaries showed no signs of development, and as far as could be determined were morphologically and histologically pupal. In contrast, the ovaries of control animals had differentiated in the manner described by King and Aggarwal (1965) although none of the eggs were covered with a chorion.

The fat body of second pupae also appeared pupal by morphological criteria. The cells of pupal fat body are held together in sheets by a connective tissue sheath. Early in adult development, this sheath breaks down and most of the fat body cells become loosened from each other. Later in adult development, the sheath reappears, and the fat body cells are again held together, this time in strands (Madhavan and Schneiderman, 1968). This sheath failed to break down in JH-injected pupae, and was retained when pupae molted into second pupae.

2. *Proteins detected in the blood of female Polyphemus pupae*

Figure 1 illustrates the protein bands that can be detected in the blood of a normal female Polyphemus pupa freshly removed from the cold. In addition to many lighter bands, whose visibility can be increased by applying larger amounts of protein and by varying the conditions of separation, the electrophoretic pattern of female blood contains two conspicuous protein bands. These are designated 4B (R_f with respect to the tracking dye = 0.47 in 4.5% gels) and 5A (R_f with respect to the tracking dye = 0.51 in 4.5% gels). Bands 4B and 5A were not resolved further in experiments which varied the gel concentrations and the amount of protein applied to each gel. For this reason, each band is thought to represent a single species of protein molecule. As seen in Figure 1, protein 4B is sex-limited. It probably corresponds to the sex-limited yolk protein antigen first described by Telfer and Williams (1953) in *Hyalophora cecropia* (their "antigen 7"). Protein 5A is not sex-limited.

3. *Changes in the concentrations of proteins 4B and 5A during development*

Densitometric determinations were made on the blood samples collected from three experimental animals and three control animals and expressed as μg. beef serum albumin equivalents. They are plotted in Figure 2, and reflect the changes in concentration of protein bands 4B and 5A. As Figure 2 shows, the concentration of protein 4B is markedly affected by juvenile hormone whereas the concentration of protein 5A is very little affected. In JH-treated pupae protein 4B may reach twice the concentration it has in control animals. Figure 3 records the ratio (protein 4B)/(protein 5A) and demonstrates that this ratio differs in experimentals and controls largely because of the changing concentration of protein 4B.

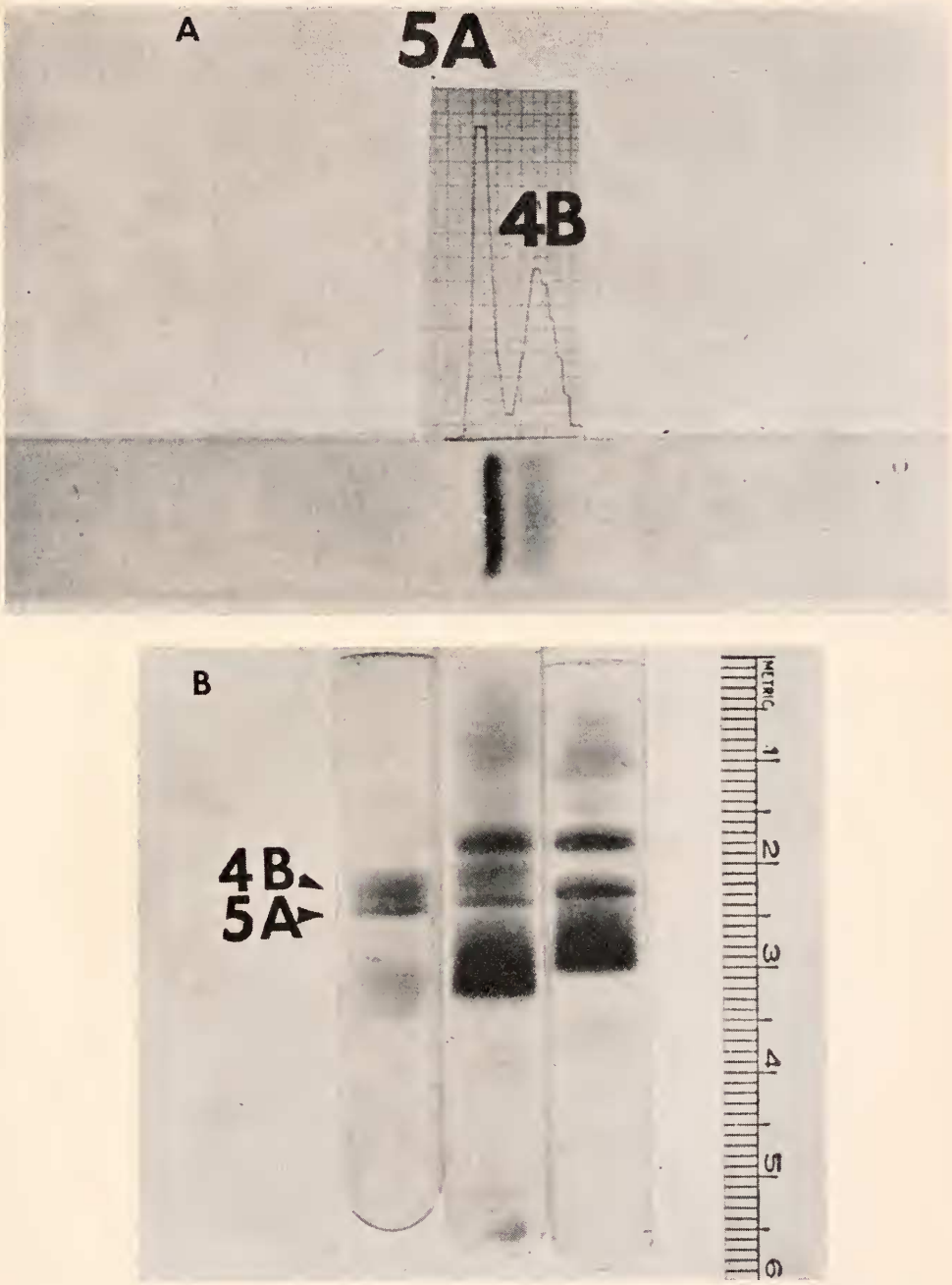

FIGURE 1. Electrophoretic separation of pupal Polyphemus blood in 4.5% acrylamide gels. A. Electrophoretic separation and densitometric recording of bands 4B and 5A in female blood. The densitometric values obtained were converted to μg. of beef serum albumin-equivalent by referring to a standard curve. B. Left to right: 0.5 μl. of female blood; 0.5 μl. of female blood + 2.0 μl. of male blood; 2.0 μl. of male blood. Band 4B is not visible in male blood.

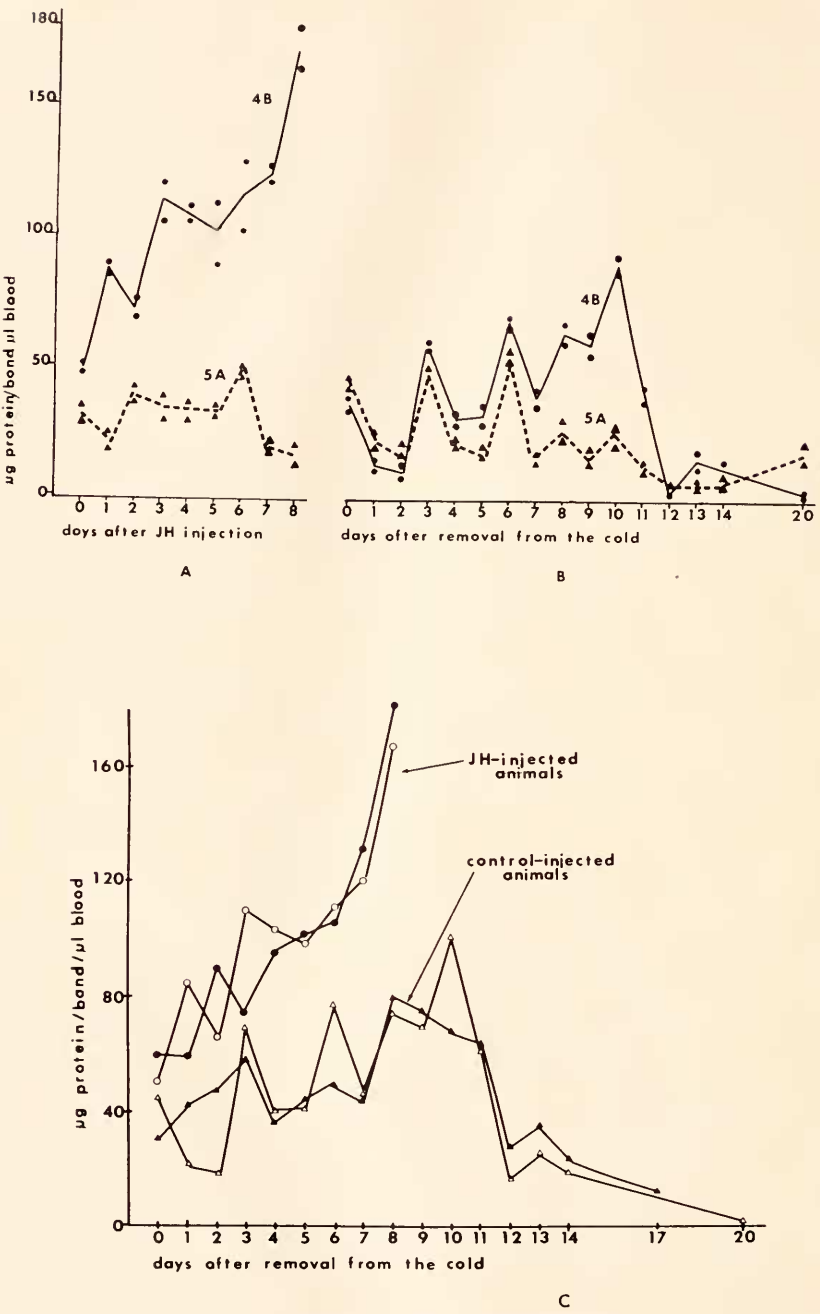

FIGURE 2. Changes in the concentrations of protein bands 4B and 5A in the blood of females during adult and second pupal development. A. Daily measurements of the concentrations of bands 4B and 5A in a typical animal injected with JH. Each point is based

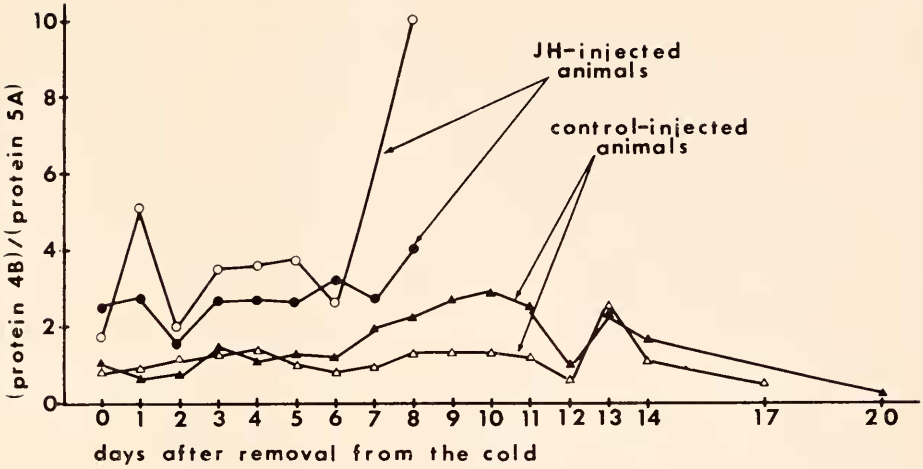

FIGURE 3. Comparison of the daily changes in the ratio of (protein 4B)/(protein 5A) in the blood of JH-injected and control animals. Each line represents one animal. Each point represents the average of the ratios of 4B to 5A.

4. *Proteins in the fat body and ovaries*

The results of electrophoretic separations of the protein in the fat body and ovaries of experimental animals are summarized in Tables I and II, and indicate the following:

1. The ovaries of adults contain proteins 4B and 5A. The ovaries of pupae and of JH-injected second pupae, in contrast, contain protein 5A, but do not contain protein 4B.

2. The blood of female pupae, 0–17-day developing adults, and JH-injected second pupae contains proteins 4B and 5A. The blood of adult females contains protein 5A, but does not contain protein 4B.

TABLE I

The distribution of protein bands 4B and 5A in the blood, fat body and ovaries of female pupae, developing adults, adults, and JH-injected second pupae

Band number	R_f in 4.5% gel	Blood				Fat body			Ovaries		
		P	DA	A	P_2	P	A	P_2	P	A	P_2
4B	0.47	+	+	0	+	0	0	0	0	+	0
5A	0.51	+	+	+	+	+	+	+	+	+	+

P = pupa; DA = 0-17-day developing adult; A = adult; P_2 = second pupa; + = present; O = absent.

on densitometric measurements of one or two separate gels. B. Daily measurements of the concentrations of bands 4B and 5A in a typical control animal. Each point is based on densitometric measurements of one or two separate gels. C. Comparison of the daily changes in the concentrations of protein 4B in the blood of JH-injected and control animals. Each line represents one animal. Each point represents the average of densitometric measurements of two or more separate gels.

3. The fat body of males and females at all stages contains protein 5A, but does not contain protein 4B.

The observation that the female fat body does not contain protein 4B prompted further experiments to establish whether the fat body synthesizes and then releases protein 4B, as has been reported for other blood protein (see Discussion). Telfer (personal communication) has obtained immunological evidence that the fat body of Cecropia synthesizes and then releases the yolk protein, antigen 7. To confirm Telfer's observations, female fat body was implanted into the abdomens of male pupae, which were then allowed to develop into adults. Protein 4B was detected in the blood of these developing adults, whereas in normal developing male adults, protein 4B was never detected (Table II). Efforts to detect the synthesis and release of protein 4B by fat body incubated *in vitro* in blood and various media failed (Blumenfeld, 1968).

TABLE II

The distribution of protein bands 4B and 5A in the blood and fat body of male pupae, adults, and JH-injected second pupae

Band number	R_f in 4.5% gel	Blood				Fat body			
		P	A	A_f	P_2	P	A	A_f	P_2
4B	0.47	0	0	+	0	0	0	0	0
5A	0.51	+	+	+	+	+	+	+	+

P = pupa; A = adult; A_f = adult derived from male pupa which had received an implant of female pupal fat body; P_2 = second pupa; + = present; 0 = absent.

DISCUSSION

1. *The fate of protein 4B*

The behavior of blood proteins in Polyphemus is best considered in relation to the behavior of the blood proteins of a closely related saturniid silkmoth, the Cecropia silkworm, which have been the subject of extensive experimental analysis. The blood of the female Cecropia pupa contains a high concentration of a specific protein antigen that is present in very low concentration in the blood of the male pupa (Telfer and Williams, 1953; Telfer, 1954). This antigen, which they called "antigen 7," is accumulated by the developing oocytes to a concentration above its level in the blood, and disappears from the blood during metamorphosis. During this time, several other blood protein antigens that appear in the oocytes are not accumulated to concentrations above their level in the blood. Antigen 7 corresponds to an electrophoretically separable protein band (Laufer, 1960). Proteins that correspond to antigen 7 are present in the blood of other saturniid moths (Laufer, 1960). Protein 4B discussed in these experiments is sex-limited, is accumulated by the developing oocytes, and appears to correspond to antigen 7.

At most stages of adult development, a change in the concentration of protein 4B is paralleled by a change in the concentration of protein 5A. Between 0 and 5 days of adult development, the concentrations of proteins 4B and 5A varied from 30 to 50 μg./μl. Between 5 and 7 days of adult development, protein 5A attained

a concentration as high as 65 μg./μl., while protein 4B attained a concentration in the blood of 100 μg./μl. The concentrations of both proteins then decreased as the accelerating demands of the maturing oocytes removed them from circulation. By 10 days of adult development, protein 5A had fallen to 35 μg./μl., while protein 4B had fallen to 25 μg./μl. After this time, they decreased further in concentration.

When one injects JH into the female pupa, one discovers that JH completely inhibits ovarian development, and that second pupal ovaries are morphologically and histologically indistinguishable from those of pupae. In most insects JH either directly or indirectly exerts a trophic effect on the oocytes and is required for both oocyte growth (see review by de Wilde, 1964) and yolk accumulation (see reviews by Telfer, 1965; Engelmann, 1968). Since extirpation of the pupal corpora allata has no obvious effect on the egg development of many non-feeding higher Lepidoptera (Williams, 1959) it is generally believed that the presence of JH during adult development is not necessary for egg development in this group. In these insects egg development occurs during adult differentiation and appears to be coupled to it. Thus, when one blocks adult differentiation with JH, one perforce blocks egg development.

Changes in the blood levels of proteins 4B and 5A are closely linked during normal adult development. When one injects JH, however, this relationship is uncoupled, and the concentrations of the two proteins diverge. In the present experiments this uncoupling was first detected seven days after JH injection. The JH injection results in a 3–4-fold increase in the titre of protein 4B from 40 to about 180 μg./μl. 8 days after injection. During this time, protein 5A remains within its normal range. Telfer (1954) ovariectomized female Cecropia pupae, and found that the level of the yolk protein in the blood of such animals rose to more than twice its level in the diapausing pupa. The rising titre of protein 4B in JH-injected female Polyphemus pupae strikingly parallels Telfer's results. Indeed, as far as protein 4B is concerned, JH appears to produce the same effects as ovariectomy.

The career of protein 4B consists of periods of synthesis, release into the blood, and accumulation by the growing oocytes. JH does not affect the synthesis or release of protein 4B, but specifically blocks the accumulation of protein 4B by the oocytes. Thus, JH profoundly upsets the career of protein 4B.

2. What tissue synthesizes and releases protein 4B?

While there is conclusive evidence that the fat body synthesizes and releases specific blood proteins (Shigematsu, 1958; Price and Bosman, 1966) it has not been proven that the fat body synthesizes and releases the yolk protein of the female. Telfer (personal communication) has concluded, from immunological analyses, that the female fat body of Cecropia was indeed the source of this protein. In the present experiments, the appearance of the yolk protein, protein 4B, in the blood of males that had received implants of female fat body confirms Telfer's findings.

JH injection prevents the breakdown of the connective tissue sheath around the fat body, but does not block the synthesis of protein 4B by the fat body cells themselves. Hence, the data suggest that as far as the synthesis of this

particular protein is concerned the pupal fat body is not a biochemical target of JH at the concentrations we employed. There is also other evidence that JH injection does not prevent an adult-specific protein from appearing in the fat body (Blumenfeld, 1968).

One wonders why the biochemical metamorphosis of the pupal fat body is not blocked by juvenile hormone whereas the metamorphosis of the integument is so profoundly affected. The answer may stem from the fact that many insect cells appear to be most sensitive to juvenile hormone when they are about to synthesize or are synthesizing DNA (*cf.* for example Krishnakumaran *et al.*, 1967, p. 43–44 ff.). Pupal fat body engages in very little DNA synthesis during the pupal-adult transformation whereas epidermal cells do engage in DNA synthesis, hence the insensitivity of pupal fat body to juvenile hormone.

This research was supported in part by research grants from the USPHS. The senior author was supported by a predoctoral traineeship in developmental biology. We wish to thank Dr. Narayan G. Patel for advice on densitometry, Dr. William H. Telfer for several valuable discussions, and Mr. Louis Martonyi for assistance with the photography. Drs. George W. Nace and A. Krishnakumaran provided helpful criticisms on the typescript.

Summary

Injection with juvenile hormone causes pupae of the Polyphemus silkmoth to undergo a pupal-pupal molt instead of a pupal-adult molt. Juvenile hormone also affects the concentration in the blood of a sex-limited blood protein, the yolk protein, which is normally synthesized by the fat body of the female pupa, released into the blood, and accumulated from the blood by the developing oocytes. When juvenile hormone is injected into a female pupa, the synthesis and release of the yolk protein are not affected. However, the oocytes do not grow and do not accumulate the yolk protein. Consequently, the concentration of the yolk protein in the blood of developing second pupae increases about four-fold. This effect becomes conspicuous seven days after juvenile hormone injection.

LITERATURE CITED

Blumenfeld, M., 1968. Effects of juvenile hormone on protein synthesis during the metamorphosis of the Polyphemus silkmoth. Ph.D. thesis, Case Western Reserve University.

Davis, B. J., 1964. Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.*, **121**: 404–427.

De Wilde, J., 1964. Reproduction–endocrine control. *In:* The Physiology of Insecta (Ed. by Rockstein) **1**: 59–90. Academic Press, New York.

Engelmann, F., 1968. Endocrine control of reproduction in insects. *Ann. Rev. Ent.*, **13**: 1–26.

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **70**: 218–225.

Gilbert, L. I., and H. A. Schneiderman, 1960. The development of a bioassay for the juvenile hormone of insects. *Trans. Amer. Micro. Soc.*, **70**: 38–67.

King, R. C., and S. K. A. Aggarwal, 1965. Oogenesis in *Hyalophora cecropia*. *Growth*, **29**: 17–83.

KRISHNAKUMARAN, A., S. J. BERRY, H. OBERLANDER AND H. A. SCHNEIDERMAN, 1967. Nucleic acid synthesis during insect development-II. Control of DNA synthesis in the Cecropia silkworm and other saturniid moths. *J. Insect Physiol.*, **13**: 1–57.

LAUFER, H., 1960. Blood proteins in insect development. *Ann. N. Y. Acad. Sci.*, **89**: 490–515.

MEYER, A. S., H. A. SCHNEIDERMAN, E. HANZMANN AND J. H. KO, 1968. The two juvenile hormones from the Cecropia silk moth. *Proc. Nat. Acad. Sci.*, **60**: 853–860.

ORNSTEIN, L., 1964. Disc electrophoresis-I. Background and theory. *Ann. N. Y. Acad. Sci.*, **121**: 321–349.

PRICE, G. M., AND T. BOSMAN, 1966. The electrophoretic separation of proteins isolated from the larva of the blowfly, *Calliphora erythrocephala*. *J. Insect Physiol.*, **12**: 741–747.

RÖLLER, H., K. H. DAHM, C. C. SWEELEY AND B. M. TROST, 1967. The structure of the juvenile hormone. *Angew. Chemie, Int., Edit.*, **6**: 179–181.

SCHMIDT, E. L., AND C. M. WILLIAMS, 1953. Physiology of insect diapause. V. Assay of the growth and differentiation hormone of Lepidoptera by the method of tissue culture. *Biol. Bull.*, **105**: 174–187.

SHIGEMATSU, H., 1958. Synthesis of blood proteins by the fat body in the silkworm, *Bombyx mori* L. *Nature*, **182**: 880–882.

TELFER, W. H., 1954. Immunological studies of insect metamorphosis. II. The role of a sex-limited blood protein in egg formation by the Cecropia silkworm. *J. Gen. Physiol.*, **37**: 539–558.

TELFER, W. H., 1965. The mechanism and control of yolk formation. *Ann. Rev. Ent.*, **10**: 161–184.

TELFER, W. H., AND C. M. WILLIAMS, 1953. Immunological studies of insect metamorphosis-I. Qualitative and quantitative description of the blood antigens of the Cecropia silkworm. *J. Gen. Physiol.*, **36**: 389–413.

WILLIAMS, C. M., 1956. The juvenile hormone of insects. *Nature*, **178**, 212–213.

WILLIAMS, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult Cecropia silkworm. *Biol. Bull.*, **116**: 323–338.